New substrates for reliable enzymes: enzymatic modification of polymers

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Recent studies clearly indicate that the modification of synthetic and natural polymers with enzymes is an environmentally friendly alternative to chemical methods using harsh conditions. New processes using lipases, proteases, nitrilases and glycosidases have been developed for the specific non-destructive functionalization of polymer surfaces. The specificity of enzymes has also been exploited in polymer synthesis; for example, lipases have been used for the production of optically active polyesters. Oxidoreductases have been used for the cross-linking and grafting of lignaceous materials and for the production of polymers from phenolics. Recent successes in this area are mainly attributable to advances in the design of reaction systems (e.g. biphasic systems and micellar solutions), while the enzymes are mainly from commercial sources.

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Abbreviations

HEC	hydroxyethylcellulose
HRP	horseradish peroxidase
PA	polyamide
PAN	polyacrylonitrile
PET	polyethyleneterphthalate
PHS	poly(4-hydroxystyrene)

Introduction

New substrates for reliable enzymes including the well studied and exploited hydrolases and oxidoreductases are described in this article. Within the class of hydrolases, the most frequently employed enzymes for enzymatic synthesis and polymer modification are glycosidases (EC 3.2.1), proteases (peptidases; EC 3.4) and lipases (carboxylic ester hydrolases; EC 3.1.1), whereas in the class of oxidoreductases, tyrosinase (EC 1.10.3.1), laccase (EC 1.10.3.2) and peroxidase (EC 1.11.1.7) have been used in the majority of reports (Figure 1). Information about the structure–function relationships of these well-studied enzymes can be found in textbooks and in the scientific literature, while their mechanisms related to polymer synthesis and modification are discussed in many papers cited in this review. There are only a few examples of enzymes from other classes that have been used in polymer modification, such as hexokinases, and these are also discussed here.

The enzymatic modification of synthetic materials has immense potential both in the functionalization of bulk materials, such as polyacrylonitrile, polyamide or polyester, and in the production of polymers for speciality applications (e.g. for the production of medical devices and electronics) [1•,2,3•,4]. The major advantages of enzymes in polymer modification compared with chemical methods are milder reaction conditions and highly specific non-destructive transformations targeted to surfaces.

The application of cellulases to improve cellulose fiber properties (textile and paper pulp) is already used in industry. Only recently, the potential of oxidative enzymes for cross-linking and functionalization of lignaceous compounds has been assessed [5••]. Laccases and peroxidases have been used both for the functionalization of lignocellulose [6] and for the polymerization of phenolics [7••]. Similarly, the enzymatic synthesis and modification of polymers are interrelated in other areas, for example, in the lipase-catalyzed ring-opening graft copolymerization of ε-caprolactone onto hydroxyethylcellulose [8]. Thus, the enzymatic synthesis of polymers is also briefly discussed here.

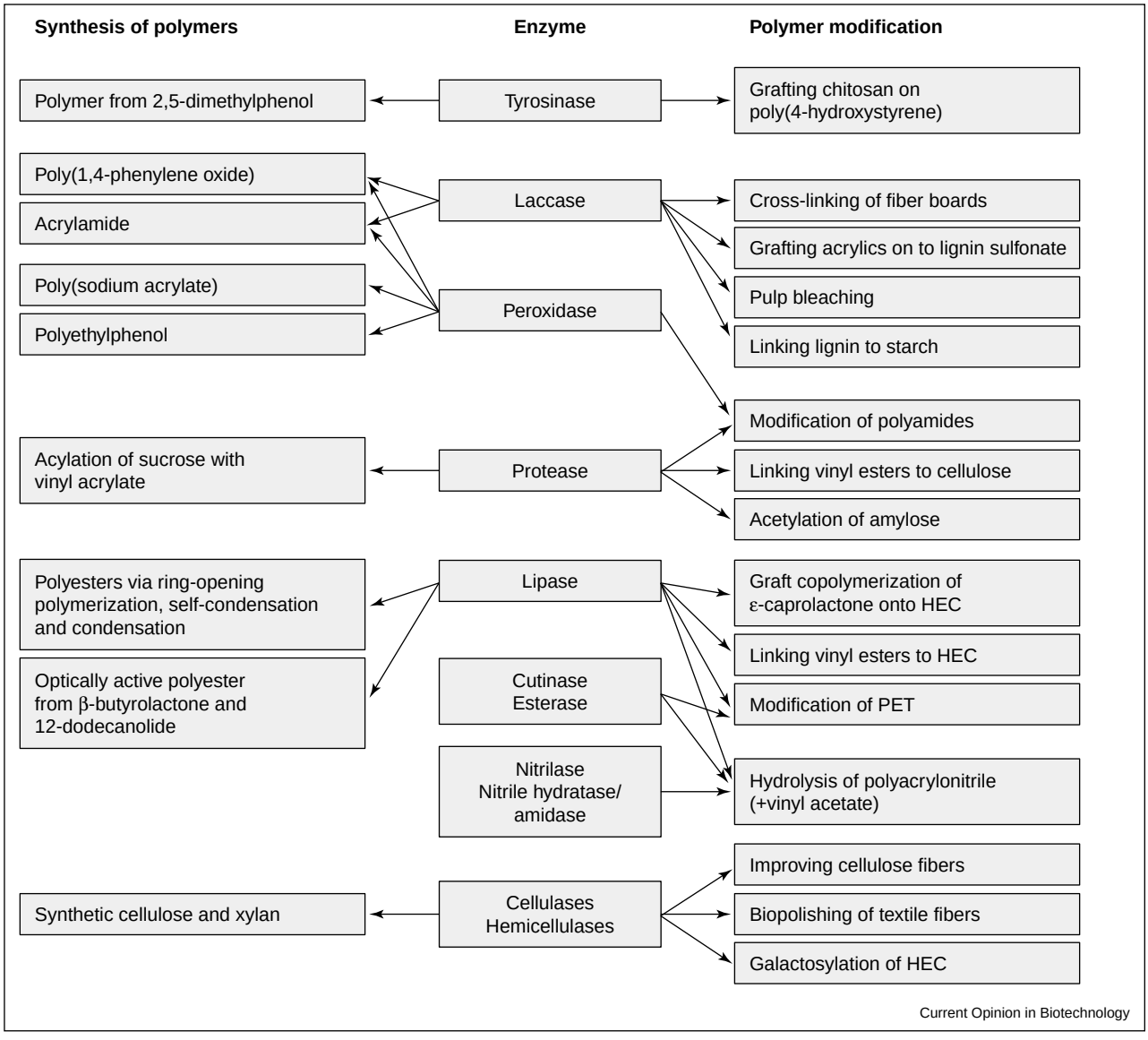
In the production of polymers, enzymes provide an environmentally friendly alternative to current processes based on harsh conditions and reactive starting materials. Furthermore, the high specificity of enzymes can be exploited in the synthesis of special materials, such as optically active polymers with potential as catalysts in asymmetric syntheses and possible roles as chiral absorbents for the separation of racemic mixtures [4,9]. Here we review recent applications of reliable enzymes for the synthesis of new substrates and discuss their future potential.

Synthetic polymers

The synthesis of polyesters with lipases

The synthesis of polyesters with lipases can be classified into ring-opening polymerization, self-condensation and condensation or transesterification reactions. In general, the design of the reaction system (e.g. the choice of solvents) seems to be crucial in determining the nature of the reaction. In the polymerization of macrolides, the reaction rates and resulting molecular weights were

Figure 1



Schematic representation of enzymes involved in the enzymatic modification and synthesis of polymers.

also dependent on the origin of the lipase used (e.g. *Pseudomonas fluorescens*, *Candida cylindracea* or porcine pancreas). Numerous examples of the lipase-catalyzed self-condensation of hydroxyesters and the condensation of dicarboxylic acids or deriviatives with diols have been reported [7••].

As mentioned previously, the potential of enzymes as highly specific catalysts can be exploited in the synthesis of optically active polymers. An optically active copolymer with an enantiomeric excess (ee) of 69% was enzymatically synthesized from β-butyrolactone and 12-dodecanolide, where (*S*)-β-butyrolactone was preferentially transformed [7••].

The synthesis of vinyl sugars with proteases

Polymers synthesized from vinyl sugars have enhanced antistatic properties, dyeability, adhesion and biocompatibility compared with conventional polymers. These polymers have potential for a number of applications; for example, in chiral separation techniques or medical devices. The production of polymers from vinyl sugars is chemically difficult to achieve, however, owing to the need for several protection and de-protection steps. Nevertheless, a monoacrylate derivative was obtained by the protease-catalyzed regioselective acylation of sucrose with vinyl acrylate, which was subsequently polymerized. Using divinyladipate and an alkaline protease from *Streptomyces* sp. the corresponding vinyl sugar esters were

synthesized with several monosaccharides including D-glucose, D-mannose, D-galactose and D-arabinose [10].

The synthesis of phenolic and acrylic polymers with oxidoreductases

Phenol-formaldehyde resins show excellent chemical, physical and mechanical properties, but alternative production processes avoiding the use of toxic formaldehyde are strongly desired [7••]. Several enzymatic methods have therefore been established for the production of resins. Poly(2,6-dimethyl-1,4-oxyphenylene) was produced from 2,6-dimethylphenol using horseraddish peroxidase (HRP) or a laccase from *Pycnoporus coccineus* [7••]. In a second example, a new crystalline polymer with a melting point above 300°C was synthesized from 2,5-dimethylphenol using tyrosinase [11]. In the conventional chemical dehalogenation and polycondensation of crystalline plastics with melting points above 300°C, high reaction temperatures are usually needed and equal molar amounts of halogenated compounds result as by-products. Using an enzymatic approach, however, polyethylphenol was synthesized with HRP in micellar solutions [12] and both the molecular weight and dispersity could be controlled by solubility parameters of the reaction medium [13].

Recently, in the presence of the mediator 2,2′-azino-di-[3-ethylbenzothiazoline-6-sulfonic acid] (ABTS), laccase was shown to catalyze the formation of copolymers of ABTS and the monomeric lignin model compounds guaiacol (2-methoxyphenol) or erol (1-(4-hydroxy-3-methoxyphenyl)-2(2-methoxyphenoxy)-propane-1,3-diol) [14]. Besides these studies on lignin modification, laccases and HRPs were also used for the production of polyacrylamide and polysodium acrylate. Both yields (>88%) and reaction rates were enhanced by the addition of surfactants or by performing reactions in concentrated emulsions [15].

Unfortunately, no data are available in the literature to compare the performance of laccases, tyrosinases and peroxidases in these processes.

Modification of synthetic polymers

The classical chemical modification of synthetic polymers requires high amounts of energy and chemicals (binders, coupling agents, etc), which are partially discharged to the environment. Furthermore, some of the substances used during the processing of fibers are released from the end-products due to weak bonding, causing serious health risks and reducing the technical life-time of the products. Polyethyleneterphthalate (PET), polyacrylonitrile (PAN) and polyamide (PA) fibers share as common features a high crystallinity and low moisture regain. Several methodologies, such as alkaline treatments that render synthetic fibers more hydrophilic, lead to the deterioration of other product properties such as irreversible yellowing of the PAN and PA fibers. The potential of microbial enzymes for the targeted surface functionalization of PET, PAN and PA has recently been assessed [16].

Polyester (polyethyleneterephthalate)

The controlled surface hydrolysis of PET facilitates the attachment of cationic compounds (e.g. dyes) or the direct application of coatings for the production of technical fabrics without the need for, or with reduced consumption of, coupling-agents. Treatment of PET with lipases has been shown to improve wetting and absorbency of PET fabrics, while strength properties were retained [17]. Other authors claimed improved stain resistance, wetting and/or dying abilities of PET fabrics treated with so-called polyesterases (lipases, esterases or cutinases) [3•]. Pilling properties of polyester fabrics were also found to be improved by treatment with enzyme preparations from *Humicola* sp., *Candida* sp. and *Pseudomonas* sp. [18]. The hydrophilicity of PET was increased by laccase treatment in combination with a mediator; however, the mechanism of this effect and the resulting chemical changes have not yet been elucidated [19].

Polyamides

Recent studies demonstrated that manganese peroxidase was able to modify the surface of PA66 and PA6 without reducing the fiber diameter [2]. Laccases in combination with a mediator have been shown to increase the hydrophilicity of PA66 fabrics [19]. Alternatively, partial hydrolysis of PA and PA oligomers has been demonstrated with proteases [16,20].

Polyacrylonitrile

Using X-ray photoelectron spectroscopy, enzyme preparations from *Rhodococcus rhodochrous* [1•], *Brevibacterium imperiale* and *Corynebacterium nitrilophilus* [21] were shown to hydrolyze PAN. Interestingly, nitrile groups of granular PANs were converted into the corresponding acids by the sequential action of nitrile hydratase and amidase from *R. rhodochrous*, while 16% of surface nitrile groups of PAN fibers were converted to the corresponding amides by the nitrile hydratase. Owing to the enzymatic modification, the acrylic fibers became more hydrophilic and the adsorption of dyes was enhanced [1•]. Many materials termed PAN actually contain about 7–10% vinyl acetate. Therefore, in addition to nitrile-hydrolyzing enzymes, esterases can also be used for the modification of these copolymers.

Other polymers

Tyrosinase has been used to graft nucleophilic compounds onto poly(4-hydroxystyrene) (PHS) in a mixture of methanol and water [22]. Aniline as a model substrate was coupled to reactive quinones resulting from the enzymatic conversion of phenolic moieties of PHS. The potential of this technique has been shown by coating PHS with chitosan. The lipase-catalyzed acylation of poly[*N*-(2-hydroxypropyl)-11-methacryloylaminoundecanamide-*co*-styrene] and the corresponding

monomer was achieved with vinyl acetate, phenyl acetate, 4-fluorophenyl acetate and phenyl stearate as acylating agents [23].

Synthesis of natural polymers

Xylan and cellulose were synthesized using endoglucanases from *Trichoderma viride* and β-xylobiosyl fluoride and β-D-cellobiosyl fluoride as substrates, respectively. Only a limited degree of polymerization was achieved in the synthesis of cellulose, owing to the low solubility of the oligomers, but larger xylan fragments were obtained. Chitin was synthesized by ring-opening polyaddition of a chitobiose oxazoline using chitinase from a *Bacillus* sp. [24].

Recently, cellulose and β-glucans were synthesized *in vitro* using cellulose and callose synthase preparations from *Rubus fruticosus* and *Arabidopsis thaliana*. However, the purification of the required enzymes, and especially of cellulose synthases, seems to be extremely difficult because of their location in plasma membranes and their low stability [25].

Modification of natural polymers

Polysaccharides

The enzymatic functionalisation of polysaccharides (e.g. regioselective acylation) has the potential to produce amphiphilic, biodegradable and biocompatible compounds for use as emulsifiers, compatibilizers, detergents and drug-delivery systems. Amylose was regioselectively acylated using proteases from *Bacillus subtilis*. This transesterification was carried out in isooctane containing the respective fatty-acid ester and a thin layer of amylose deposited onto zinc-selenide slides [26]. In another example, lignin was coupled to starch using laccase, decreasing the viscosity of the resulting material to less than 10% that of native starch [27].

Ring-opening graft copolymerization of ϵ-caprolactone onto hydroxyethylcellulose (HEC) was carried out using a lipase from porcine pancreas [8]. Succinic-HEC, stearoyl-HEC and acetylated HEC have been produced using succinic acid, vinyl stearate and vinyl acetate, respectively, and a lipase from *Pseudomonas cepacia*. Galactose was also successfully linked to HEC by a transglycosylation reaction from lactose using a galactosidase from *Aspergillus oryzae* [28].

As in the modification of HEC, vinyl esters have been used as substrates to produce cellulose acrylate and proprionate (e.g. for use in cotton, rayon and filter paper). This reaction was carried out in pyridine using subtilisin as biocatalyst [17]. Currently, harsh reaction conditions and low yields limit the potential of these otherwise very promising biotransformations of cellulose. Recently, cotton cellulose has been phosphorylated using a hexokinase from baker's yeast; ATP was the phosphoryl donor in the reaction and primary hydroxyl groups of cellulose were phosphorylated. Despite the high cost of ATP, the process looks promising as the phosphorylation of cellulose enhanced dyeability and flame resistance [29].

Lignocellulose materials

Cellulases and hemicellulases can improve drainage of recycled pulps by hydrolyzing the amorphous hydrophilic cellulose, which is the main constituent of the fines (i.e. the fraction of pulp that passes a 76 μm screen) formed during refining [30]. Endoglucanase treatment can decrease viscosity and increase the alkaline solubility of dissolving pulp [31], whereas the removal of hemicellulose can be enhanced by the application of hemicellulases [32]. Additionally, hemicellulases can enhance the removal of lignin in pulp bleaching. Hemicellulases and cellulases from different origins and families show remarkable differences in substrate specificities, and their structure–function relationships have been studied extensively [33–35]. In the processing of cotton, viscose or lyocell, cellulases are used to modify fiber and fabric surfaces, for example, by removing microfibrils and fuzz fiber or by inducing fibrillation. The bio-stone washing of denim fabrics is based on enzymatic fibrillation, which releases indigo entrapped inside the fibers [36].

Lignin

The use of laccase in combination with mediators for the delignification of pulps was introduced about ten years ago. More recently, the potential of this enzyme for cross-linking and functionalizing lignaceous compounds was discovered [5••]. Laccases can be used for bonding of fiberboards, particle boards, paper boards and kraft-liner boards. Recently, it was shown that the strength properties of laccase-bonded fiberboards are comparable to boards bonded using a urea-formaldehyde adhesive. It is likely that the enhanced bonding is caused by covalent bonds between fibers or by an adhesive effect of polymerized loosely associated lignin [6].

Laccase has also been used for the chemo-enzymatic synthesis of lignin graft-copolymers. It was suggested that during grafting of lignin sulfonate and acrylic monomers in the presence of *t*-butylhydroperoxide, the latter is probably reduced to alcoxy radicals while phenoxy radicals are oxidized to quinones. Alcoxy or peroxy radicals seem to be responsible for starting the sidechain polymerization of acrylic monomer radicals, whereas termination of the reaction is caused by the coupling of growing acrylic chains and phenoxy radicals produced by laccase [37•].

Conclusions

The well-studied and exploited hydrolases and oxidoreductases not only have immense potential for the modification of natural polymers, such as cellulose or lignin and their derivatives, acting on their natural substrates, but also show promise for the functionalization of synthetic

materials. Enzyme engineering combined with sophisticated analysis techniques and screening assays should lead to the development of more efficient enzymes with higher turnover rates and stabilities in organic solvents. However, reaction engineering will still remain an important factor in the design of enzymatic modification reactions of (mostly water insoluble) polymers.

References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Tauber MM, Cavaco-Paulo A, Robra K-H, Gübitz GM:
• **Nitrile hydratase and amidase from *Rhodococcus rhodochrous* hydrolyse acrylic fibers and granulates**. *Appl Environ Microbiol* 2000, **66**:1634-1638.

Using X-ray photoelectron spectroscopy (XPS) staining methods and quantification of ammonia, released the authors show for the first time that nitrile groups of polyacrylonitriles are hydrolyzed by enzymes from *R. rhodochrous*.

2. Deguchi T, Kitaoka Y, Kakezawa M, Nishida T: **Purification and characterization of a nylon-degrading enzyme**. *Appl Environ Microbiol* 1998, **64**:1366-1371.

3. Yoon M-Y, Kellis J, Poulose AJ: **Enzymatic modification of**
• **polyester**. *AATCC Review* 2002, **2**:33-36.

Various enzymes termed polyesterases were found to hydrolyze PET improving stain resistance and the ability to wet and dye PET fabrics.

4. Ritter H: **Functionalized polymers via enzymic synthesis**. In *Desk Reference of Functional Polymers*. Edited by Arshady R. Washington: American Chemical Society; 1997:103-113.

5. Grönqvist S, Suurnäkki A, Niku-Paavola ML, Kruus K, Buchert J,
•• Viikari L: **Lignocellulose processing with oxidative enzymes**. *ACS Symp Ser* 2003, **855**: in press.

This review gives a comprehensive overview of the potential of oxidoreductases in the modification of lignocellulose, such as lignin activation or fiber cross-linking.

6. Felby C, Hassingboe J, Lund M: **Pilot-scale production of fiberboards made by laccase oxidized wood fibers: board properties and evidence for cross-linking of lignin**. *Enzyme Microb Technol* 2002, **31**:736-741.

7. Uyama H, Kobayashi S: **Enzyme-catalyzed polymerization**
•• **to functional polymers**. *J Mol Catal B Enzym* 2002, **19-20**:117-127.

Both new routes for the synthesis of polyesters — with hydrolases and the polymerization of phenolics with laccases and HRP — are covered.

8. Li J, Xie W, Cheng HN, Nickol RG, Wang PG: **Polycaprolactone-modified hydroxyethylcellulose films prepared by lipase-catalyzed ring-opening polymerization**. *Macromolecules* 1999, **32**:2789-2792.

9. Cheng HN, Gu Q-M: **Enzyme-catalyzed reactions of polysaccharides**. *ACS Symp Ser* 2003, **840**:203-216.

10. Dordick JS: **Enzymatic and chemoenzymatic approaches to polymer synthesis**. *Trends Biotechnol* 1992, **10**:287-293.

11. Kobayashi S, Higashimura H: **Oxidative polymerization of phenols revisited**. *Prog Polym Sci* 2003, **28**:1015-1048.

12. Rao AM, John VT, Gonzalez RD, Akkara JA, Kaplan DL: **Catalytic and interfacial aspects of enzymatic polymer synthesis in reversed micellar systems**. *Biotechnol Bioeng* 1993, **41**:531-540.

13. Ayyagari MS, Marx KA, Tripathy SK, Akkara JA, Kaplan DL: **Controlled free-radical polymerization of phenol derivatives by enzyme-catalyzed reactions in organic solvents**. *Macromolecules* 1995, **28**:5192-5197.

14. Rittstieg K, Suurnäkki A, Suortti T, Kruus K, Gübitz GM, Buchert J: **Polymerization of guaiacol and of a phenolic β-O-4 substructure by *Trametes hirsuta* laccase in the presence of ABTS**. *Biotechnol Prog* 2003, in press.

15. Kalra B, Gross RA: **HRP-mediated polymerizations of acrylamide and sodium acrylate**. *Green Chem* 2002, **4**:174-178.

16. Fischer-Colbrie G, Heumann S, Cavaco-Paulo A, Gübitz GM: **Enzymatic modification of synthetic fibres**. *Abstracts of Papers, 225th ACS National Meeting: 2003 Mar 23–27; New Orleans, USA*; Abstract: *Cell* 111.

17. Xie J, Hsieh YL: **Modification of cellulose solids by enzyme catalysed transesterification with vinyl esters in anhydrous organic solvents**. *ACS Symp Ser* 2003, **840**:217-230.

18. Andersen X, Bente Konggaard X, Borch X, Kim X, Abo X, Masanobu X, Damgaard X, Bo X: **Method of treating polyester fabrics**. 1999 US Patent 5,997,584:1-20.

19. Miettinen-Oinonen A, Silvennoinen M, Nousiainen P, Buchert J: *Modification of Synthetic Fibres with Laccase*. *Proceedings of the Second International Symposium on Biotechnology in Textiles: 2002 Apr 3–6; Athens*, 2003; Abstract: 024.

20. Kakudo S, Negoro S, Urabe I, Okada H: **Nylon oligomer degradation gene, *nylC*, on plasmid pOAD2 from Flavobacterium strain encodes endo-type 6-aminohexanoate oligomer hydrolase: purification and characterization of the *nylC* gene product**. *Appl Environ Microbiol* 1993, **59**:3978-3980.

21. Battistel E, Morra M, Marinetti M: **Enzymatic surface modification of acrylonitrile fibers**. *Appl Surf Sci* 2001, **177**:32-41.

22. Shao L, Kumar G, Lenhart JL, Smith PJ, Payne GF: **Enzymatic modification of the synthetic polymer polyhydroxystyrene**. *Enzyme Microb Technol* 1999, **25**:660-668.

23. Pavel K, Ritter H: **Lipase-catalyzed acylation of comb-like methacrylic polymers containing HO groups in the side chains**. *Makromol Chem* 1992, **193**:323-328.

24. Kobayashi S, Sakamoto J, Kimura S: ***In vitro* synthesis of cellulose and related polysaccharides**. *Prog Polym Sci* 2001, **26**:1525-1560.

25. Him JLK, Chanzy H, Pelosi L, Putaux JL, Bulone V: **Recent developments in the field of *in vitro* biosynthesis of plant β-glucans**. *ACS Symp Ser* 2003, **840**:65-77.

26. Akkara JA, Ayyagari MSR, Bruno FF: **Enzymatic synthesis and modification of polymers in nonaqueous solvents**. *Trends Biotechnol* 1999, **17**:67-73.

27. Lutkemeyer S, Huttermann A: **Enzymatic derivatization of starch with lignin**. *Abstracts of Papers, 225th ACS National Meeting; 2003 Mar 23-27; New Orleans, USA*. Cell 10; 2003.

28. Li J, Cheng HN, Nickol RG, Wang PG: **Enzymatic modification of hydroxyethylcellulose by transgalactosylation with β-galactosidases**. *Carbohydr Res* 1999, **316**:133-137.

29. Tzanov T, Stamenova M, Cavaco-Paulo A: **Phosphorylation of cotton cellulose with Baker's yeast hexokinase**. *Macromol Rapid Commun* 2002, **23**:962-964.

30. Oksanen T, Pere J, Paavilainen L, Buchert J, Viikari L: **Treatment of recycled kraft pulps with *Trichoderma reesei* hemicellulases and cellulases**. *J Biotechnol* 2000, **78**:39-48.

31. Rahkamo L, Viikari L, Buchert J: **Enzymatic and alkaline treatments of hardwood dissolving pulp**. *Cellulose* 1998, **5**:79-88.

32. Gübitz GM, Stebbing D, Johansson CI, Saddler JN: **Lignin-carbohydrate complexes restrict enzymatic solubilization of mannan and xylan from dissoving pulp**. *Appl Microbiol Biotechnol* 1998, **50**:390-395.

33. Gübitz GM, Haltrich D, Latal B, Steiner W: **Mode of depolymerization of hemicellulose by various mannanases and xylanases in relation to their ability to bleach softwood pulp**. *Appl Microbiol Biotechnol* 1997, **47**:658-662.

34. Schmidt A, Gübitz GM, Kratky C: **Xylan binding subsite mapping in the xylanase from *Penicillium simplicissimum* using xylooligosachharides as cryo-protectant**. *Biochemistry* 1999, **38**:2403-2412.

35. Henrissat B, Davies G: **Structural and sequence-based classification of glycoside hydrolases**. *Curr Opin Struct Biol* 1997, **7**:637-644.

36. Cavaco-Paulo A, Morgado J, Andreaus J, Kilburn D: **Interactions of cotton with CBD peptides**. *Enzyme Microb Technol* 1999, **25**:639-643.

37. Mai C, Schormann W, Huttermann A, Kappl R, Huttermann J:
• **The influence of laccase on the chemo-enzymatic synthesis of lignin graft-copolymers**. *Enzyme Microb Technol* 2002, **30**:66-72.
On the basis of sophisticated analysis techniques, the mechanism of laccase-catalyzed oxidation of lignin sulfonate in the presence of *t*-butylhydroperoxide is elucidated.